US Patent Application No: **09/756,185**

"Component B as angiogenic agent in combination with human growth factors"

Applicant: Borelli, Donini and Ziche

Declaration of Professor Marina Ziche

I, Marina Ziche, do hereby declare as follows:

1. I am currently employed as Professor of Pharmacology, at the University of Siena, Siena, Italy. I have worked in the field of molecular pharmacology since 1979. For the past 20 years, much of my work has concerned the study of angiogenesis and the factors that influence it. I have published over 100 refereed papers and articles on the topic of angiogenesis. My *curriculum vitae* is attached as Exhibit A.

2. I am named as co-inventor on US patent application no. 09/756,185 ("the Application"). I was involved in designing the experiments reported therein on pages 3 to 9, and it was in my laboratory and under my supervision that the work was carried out.

3. In the experiments reported in the Application, angiogenesis was assessed using the rabbit cornea assay. In this assay, micro-pockets are produced in the cornea of an anaesthetised rabbit. Pellets of the test compounds, prepared with a slow-release polymer (Elvax-40) are placed in the micro-pockets. Angiogenesis is recorded daily by examining the cornea under a slit lamp stereomicroscope. The number of implants exhibiting neovascularisation and the density of new vessels are recorded, permitting the assignment of an "angiogenic score".

4. On page 6 of the Application, experiments are reported in which Component B and basic fibroblast growth factor (bFGF) are implanted in corneal micro-pockets. To assess the synergism between Component B and bFGF, the compounds were implanted both alone and together. The results are shown in Figures 4A and 4B of the Application, which show angiogenic score progression from 0 to 12 days after implantation.

5. I have prepared revised versions of Figures 4A and 4B, which are attached as Exhibit B. The revised figures show the results of the same experiments reported in the Application, with error bars.

6. Revised Figures 4A and 4B show the angiogenic score over time when 500 ng of Component B [CB] were implanted [open squares], and when 100 ng of bFGF were implanted [filled diamonds], each agent being implanted in a different cornea (n=7). Each compound was implanted as a single pellet. These results show the effect of each agent acting alone.

7. Revised Figure 4A also shows the effect when Component B and bFGF were implanted together imbedded in a single pellet containing both agents [open circles]. The combined treatment invariably resulted in a higher angiogenic response relative to the response obtained with single molecules. The score values were significantly higher between days 5 and 12 [n=7, $P<0.01$, asterisks in Figure 4A].

8. Revised Figure 4B compares the effect exerted by Component B and bFGF released from single pellets with that exhibited by the combination of Component B and bFGF released simultaneously from two adjacent pellets. The combination of the two agents results in a greater angiogenic response, which becomes statistically different from a single molecule response at day 5 and 7 [n=7, $P<0.01$, asterisks in Figure 4B]. It is noted that the total doses of Component B and bFGF were 500 ng and 100 ng, respectively, in all cases.

9. The procedure using two pellets [Figure 4B] provides higher angiogenic response than when Component B and bFGF are combined in one pellet. This is probably due to interference and/or competition in the release rate of the two molecules when they are embedded in the same pellet. The two-pellet experiment provides a "clearer" experimental condition.

10. Regardless of whether the two agents are in two adjacent pellets or a single pellet, the response to the combined treatment is greater than the sum obtained with single molecules. As an example, at day 7, the sum of angiogenic score elicited by single molecules is 1.3, while that of the combination treatment is 3 and 4.5 with one and two tablets, respectively. The synergism between Component B and bFGF is clearly demonstrated.

11. Synergism was also observed when Component B was combined with another growth factor, i.e. VEGF. These results are reported in Table 3 of the Application. The angiogenic response to a combination of Component B [400 ng] and VEGF [100 ng] yields an angiogenic response greater [$P<0.01$] than that obtained with single molecules.

12. I have been asked to comment on the statistical significance of the results shown in Figures 4A and 4B. As mentioned in the Application, at page 5, lines 5-9, results were expressed as means for n=7 implants. Multiple comparisons were performed by one-way ANOVA and individual differences were tested by Fisher's test after the demonstration of significant intergroup differences by ANOVA. A P-value of less than 0.05 was taken as significant.

13. A P-value of < 0.01, as reported in the two above-described experiments, provides evidence for the synergy of Component B and bFGF. This synergism was noteworthy, and we proceeded to seek patent protection for a method of promoting angiogenesis using Component B and a human growth factor.

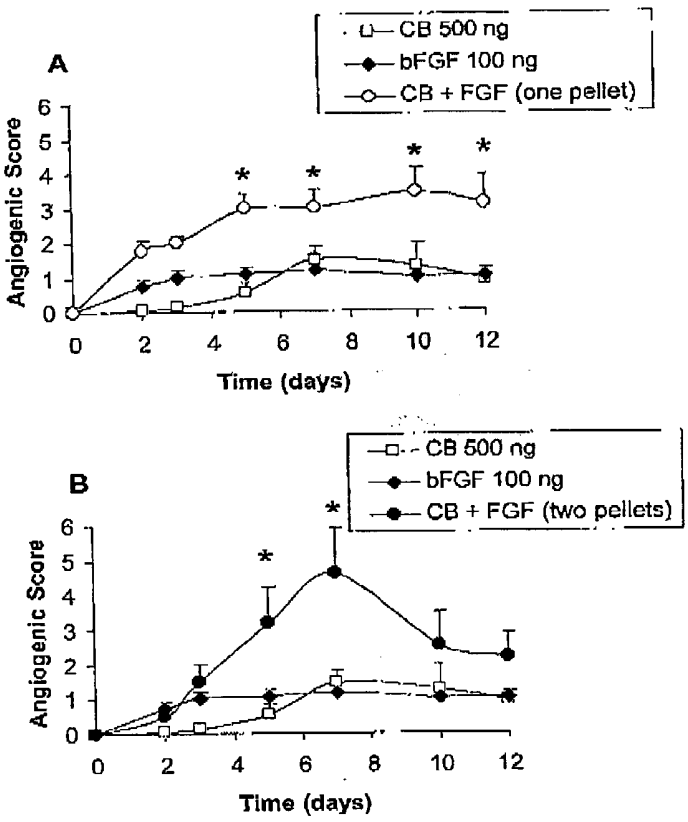
Declared at Siena, Italy

Date:29/06/2004

Marina Ziche, Ph.D.

Exhibit A: *curriculum vitae* of Marina Ziche
Exhibit B: revised Figures 4A and 4B

Fig. 4



*P<0.01 vs either CB or bFGF alone (Student t test for grouped data).

CURRICULUM VITAE

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Professional History:

1977	M.D. degree, School of Medicine, Univ. of Florence, Italy.
1979-82	Fogarty Fellow, National Cancer Institute at NIH, Laboratory of Pathophysiology, Bethesda, MD, USA.
1985-1986	Visiting Scientist, Weizmann Institute of Science, Dept. of Hormone Research, Rehovot, Israel.
1986	Visiting Scientist, Karolinska Institutet, Dept. of Pharmacology, Stockholm, Sweden.
1990-92	Visiting Professor, Microcirculation Research Institute, Dept. of Medical Physiology, Texas A&M University, College Station, TX, USA
1998-2001	Associate Professor of Pharmacology, University of Siena, Institute of Pharmacology, Italy
2000-2001	Visiting Professor, Dept. Pharmacology, U.T. South Western Medical School, Dallas, TX USA
2001-present	Full Professor of Pharmacology, School of Pharmacy, University of Siena, Italy

Relevant experience and expertise:

The laboratory of Prof. Marina Ziche focuses on research of the endothelium with particular emphasis on angiogenesis, one of the principal functions of this tissue. Angiogenesis, a process which leads to formation of new vessels, has a relevant physiological role during development and in adult life. The involvement of angiogenesis in pathologies, particularly cancer, but also cardiovascular and neurodegenerative diseases, has sparked an intense research interest in this phenomenon.
The laboratory of Prof. Ziche has contributed to research on angiogenesis by characterising molecules (growth factors, cytokines...) which exert important effects on the formation of new blood vessels in pathologies such as cancer and cardiovascular diseases. Other significant contributions deal with the elucidation of signalling pathways involved in angiogenesis (NO, cGMP) and with the control of the acquisition of the angiogenetic phenotype by endothelial cells.

Ongoing research projects:
-Studies on angiosuppressive molecules and their exploitation as antitumor agents. This includes also novel strategies based on the interplay of NOS and COX pathways, their suppression by specific inhibitors and their relevance for colon cancer.
-Cardiovascular pathologies caused by endothelial dysfunction (ischemia and heart failure) with emphasis on growth factors and compounds able to restore the integrity of the endothelium.
-Nanostructured biomaterials which support endothelial cell growth to be used as medical devices in cardiovascular and bone diseases.
-Study of the endothelium as a barrier during parasitic invasion, and its interplay with inflammatory cells.
-Angiogenesis in neurodegenerative pathologies and in gene-linked diseases.

These studies are grounded on a long established repertoire of cell biology, biochemical and molecular biology techniques (quantitative RT-PCR). In addition the laboratory has a solid experience on ex-vivo and in vivo techniques to assess angiogenesis (rabbit cornea, mouse matrigel plug and vessels sprouting in gels), tumor growth (immunodeficient mice), and cardiovascular functions (aorta rings and isolated heart).

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